

AMENDMENTS

RECEIVED
JAN 15 2004
TECH CENTER 1600/2900

Amendments to the Specification:

Please amend the substitute specification as indicated:

Page 17, Line 21:

[Figure 14 shows the results of Northern blot analysis of the expression of sense and anti-sense VEGF RNA in Ad.VAI.VEGFS and Ad VALVEGFAS transduced human embryonal kidney cells (293 cells).

Figure 15 shows the results of Northern blot analysis of the expression of sense and anti-sense VEGF RNA in Ad.VAI.VEGFS and AD VAI.VEGFAS transduced retinal pigment epithelial cells (RPE 51).]

Page 52, Line 15:

Example 19: **Expression of VEGF Antisense mRNA fragments
by Ad,VAI.AVEGF recombinant adenovirus**

Generation of recombinant adenoviruses expressing VA1-ratVEGF antisense RNAs. The Ad2 virus-associated RNA (VA1 RNA) was chosen to produce the antisense rat VEGF RNA structures. VA1 is a simple gene containing two intragenic promoter regions, namely box A and box B, and is transcribed by RNA polymerase III. Other RNA polymerase III-transcribed genes include those for tRNAs and 5SrRNAs, which are synthesised in large amounts and in most cell types (reviewed in Ciliberto et al, 1983). The VA1 RNA is thought to maintain a secondary structure consisting of two imperfect stems joined at a more complex and functional central domain (Ghadge et al, 1994). By cloning short antisense VEGF sequences into the loop at the end of one imperfect stem hybrid, RNA structures were produced as detailed below.

b) Cloning into pVA1

pVA1 is an expression vector containing the VA1 gene cloned into the SmaI site of pEMBL9, and was provided by A. Nichols. A BamHI site immediately downstream of box B was used for cloning in the antisense VEGF sequences. A 130bp rat VEGF fragment, corresponding to -61 to +69bp relative to the adenosine of the ATG start codon of the rat VEGF cDNA (see Genbank accession numbers U22373 and M32167) was generated by the reverse transcriptase-polymerase chain reaction (RT-PCR). The source of RNA was rat RPE cells that had been subjected to 24 hours in hypoxic conditions (2% O₂ in a Sanyo O₂/CO₂ incubator). The PCR products were cloned into the BamHI site of pVA1. The clones were subsequently sequenced for identification, Taq-induced errors and sequence orientation using dye-terminator chemistry (Perkin-Elmer, Foster City, CA) on an ABI 310 DNA sequencer. It was noted that there were three base pair differences to the published sequence (Levy et al., 1995). Both antisense and sense direction clones were isolated.

A 24bp fragment of rat VEGF, corresponding to -2 to +22bp relative to the ATG start codon was generated by annealing two specific oligonucleotides that generated BamHI sticky ends and cloning the product into the BamHI site of pVA 1. Again the orientation was determined by DNA sequencing and both antisense and sense clones isolated.

c) Generation of Adenoviruses Containing VA1-rVEGF Constructs

In order to generate adenoviruses the pVA1 constructs were sub-cloned into the vector pDE1splA (Microbix Biosystemms Inc., Ontario, Canada). This vector carries the necessary adenovirus sequences required for homologous recombination between the viral backbone and the plasmid. The pVA 1 constructs were digested with EcoRI and XbaI (Promega Corporation, Madison WI) and then directionally cloned into the multiple cloning site in pDE1splA. The new plasmids were amplified and purified by Qiagen columns (Qiagen, Hilden, Germany). The pDE1splA VA1-rVEGF plasmids were then cotransfected with Clal (Promega Corporation, Madison, WI) digested adenovirus E1-E3 deletion mutant d1324 DNA (provided by M. Perricaudet) into the human embryonal kidney cell line, 293 (Microbix Biosystems Inc., Ontario, Canada) using the calcium phosphate precipitation

method (Hilt et al, 1994). Four days later the cells were lysed by repeated cycles of freeze/thaw, and a small amount of the lysate was replated on fresh 293 cells seeded into 96 well plates. Wells showing cytopathic effect after 7 to 10 days were isolated, expanded and the DNA screened, by restriction mapping and hybridisation to radiolabelled specific oligonucleotides for successful homologous recombination. Those viruses selected for further use were then cloned by limiting dilution on monolayer 293 cells and amplified in order to generate a viral stock. Generally a viral stock was made from infecting 48 x 150cm² flasks of monolayer 293 cells. 48 hours later the cells were harvested into a small volume of 20mM Tris.Cl pH 8.0. Following several cycles of freeze/thaw the cellular debris was removed by extraction with an equal volume of trichlorotrifluoroethane (Sigma Chemical Co., St Louis, MO). The viral particles were banded by CsCl density ultracentrifugation (Hitt et al, 1994) and then dialysed overnight at 4°C against phosphate buffered saline. The viral stocks were titrated by limiting dilution on 293 cells seeded in 96 well plates.

**d) Infection of 293 Cells and RPE Cells with
AdVAIvegfl30S and AdVAIvegfl30AS**

293 cells were infected with a low multiplicity of infection (MOI) until a cytopathic effect was observed. Human RPE 51 cells were infected with an MOI of 10 and 100 with AdVAIvegfl30S and AdVAIvegfl30AS for 48 hours. RNA was isolated using Trizol (Gibco-BRL, Grand Island NY), separated by formaldehyde gel electrophoresis, transferred to ZetaProbe GT membrane (BioRad, Hercules, CA), and probed with radiolabelled oligonucleotides specific for either the sense or antisense RNA species. In both cell types the viruses were shown to be capable of producing the desired VA 1-rVEGF RNA molecules (Figures 14 and 15).

Example 20: Anti-Sense DNA Mediated Transcription Regulation

A number of alternative sites are proposed here to attempt to control the expression of the VEGF gene. These principally involve targeting regions in the 5' and 3' untranslated regions (UTR) of VEGF that have been identified to have roles in the transcriptional and post-transcriptional regulation of this molecule.

It has been found that hypoxia increases the expression of VEGF, a situation which is replicated in vivo and results in numerous disease conditions. The increase in VEGF expression by hypoxia can be accounted for by two main mechanisms: firstly, an increase in the rate of transcription, and secondly an increase in the stability of the mRNA produced (Shima et al, 1995; Levy et al, 1995).

a) HIF-1/Epo/AP-1 Enhancer

The most significant area thought to be responsible for the enhanced transcription rate contains a near consensus sequence for Hypoxia Inducible Factor-1 (HIF-1), followed closely by a region very similar in sequence to a 5 base pair enhancer element, both of which are found in the 3' untranslated region of the erythropoietin gene (Levy et al, 1995). Erythropoietin (Epo) is also known to be strongly regulated by hypoxia, and the same region of 5'UTR of VEGF has been mapped by others (Liu et al, 1995). Also closely associated with these motifs in the VEGF 5' region is a single consensus Activator Protein-1 (AP-1) binding site, which is conserved in human, rat and mouse VEGF sequences. The AP-1 transcription factors are members of the c jun and c-fos family, which are also upregulated by hypoxia, and bind as heterodimers to the AP-1 binding sites. Taken together these sites represent a strong candidate region for potential therapeutic intervention by oligonucleotide molecules.

The HIF-1/Epo/AP-1 enhancer region of human VEGF, shown below, is located between positions 1388 to 1432 (GenBank accession number M63971), or is positioned relative to the coding region between positions -2013 to -1969.

HIF-1	Epo1	AP-1
5' - CCAGACTCCACAGTGCAT <u>TACGTGGGCTCC</u> <u>AACAGGTCCTCTTCCCTCC</u> CAGTCACT <u>TGACTAA</u> CCCCGGAACACACA (SEQ. ID NO:3)		

It is likely to be of greater importance to target oligonucleotides to the HIF-1 and Epo sites, since it has been shown that hypoxic induction of VEGF transcription can be independent of a functional AP-1 site (Finkenzeller et al, 1995). Since the HIF-1/Epo/AP-1 region is a likely enhancer element for the start of the transcription process, it is possible that binding of oligonucleotides to the target DNA will have a potential effect in preventing effective transcription. An alternative strategy is to prevent binding of the trans acting

enhancer element(s) by competitive binding of the enhancer proteins using oligonucleotides, as demonstrated by Levy et al, (1995), although excess quantities may be necessary for such competitive inhibition.

Possible oligonucleotide sequences are designed within this region and are either complementary to the upper strand (to bind to the DNA itself) or the same as the upper strand (which will bind to the lower strand and also potentially compete with the enhancer proteins for binding).

b) SP-1 Sites

An alternative position of interest is the series of three adjacent SP-1 sites located at positions 2278 to 2310 (according to GenBank Accession Number M63971) or at positions -1123 to -1091 upstream of the ATG codon of the human VEGF coding region. The location of these three adjacent SP-1 sites approximately 50 bp upstream of the identified transcription start site (Levy et al, 1995) suggests that this region may play a potential role in transcriptional regulation.

The region encompassing the SP-1 sites and the transcription start site is shown below.

```

          SP-1      SP-1      SP-1
5'
CCTGTCCCGCCCCCGGGCGGGCCGGGGCGGGGTCCCGGCGGGCGGAGCCATGCGCCCCCCCCCTTTTAAAAAGTCGGCTGGTAGCGGG
GAGGATCGCGGAGGCTTGGGGCAGCCGGGTAGC 3' (SEQ. ID NO:4)
Transcripti
on start
site

```

Oligonucleotides are designed around this region to hybridise either to the upper strand and lower strands that would prevent binding of the SP-1 protein.

A fourth SP-1 site at position 2883 to 2888, shown below, located between the transcription start site, shown above, and at the ATG translation initiation site, is also useful to target in combination with the other localised SP-1 sites to help to inhibit transcription.

```

          SP-1
5'
CTGACGGAGAGACAGACAGACACCGCCCCAGCCCCAGCTACCACTCCTCCCCGGCCGG (SEQ. ID NO:5)

```

c) Transcription Start Site

The design of oligonucleotides around the transcription start site shown previously provides further possible candidates for potential therapeutic intervention by potentially inhibiting the commencement of the transcription process, particularly in the region upstream of the start site where the RNA polymerase will bind to the DNA strand.

d) AP-2 Site

A single Activator Protein-2 (AP-2) site located at position 3265 to 3274 (or -136 to -127 relative to the ATG start codon) is another potential site for oligonucleotide targeting. Activator protein-2 transcription factors are trans acting proteins which bind at this site and are responsive to cAMP levels. Targeting oligonucleotides to the AP-2 site, shown below, may also prevent or block transcription factor binding and thus inhibit transcription of VEGF.

5' AP-2
TGCGCAGACAGTGCTCCAGCCGCGCGCTTCCCCAGGCCCTGGCCCCGGGCCTCGGGCCGGGAGGAAGA (SEQ. ID NO: 6)

Oligonucleotides are synthesized to hybridise to both the upper and lower strands of the DNA to test the effectiveness of each alternative to inhibit AP-2 binding.

e) AU-Rich Sequences in 3' UTR

The 3' UTR of numerous short lived mRNA's have regions of AU-rich sequences. A particular consensus nonameric sequence has been identified: UUAUUUA(T/A)(T/A), which correlates with the unstable nature of these messages (Zubiaga et al, 1995). The presence of multiples of this motif is strongly indicative of increased instability, which is thought to be achieved by deadenylation of the polyA tail of the mRNA. VEGF contains two such nonameric instability sequences, in addition to numerous 5-nucleotide core sequence units (AUUUA). Removal of these sequences results in increased message stability and it is possible that these regions can act either by a cis or trans mechanism. These sequences may affect secondary structure formation and mediate changes in mRNA stability; proteins present in hypoxic cell extracts, which have been mapped to the

same area, have also been demonstrated to increase the stability of the message. To block the increased message stability induced by hypoxic conditions the approach is to prevent the secondary structure formation or to block binding of the trans acting factors, by targeting the mRNA with antisense oligonucleotides. An alternative approach is to block the binding of the protein by providing an excess of the sequence at which the protein binds, which in this case encompasses the nonameric AU-rich consensus motif.

The AU-rich instability elements are shown below, and are positioned at 1223 to 1231 (A) and 1726 to 1734 bases (B) respectively downstream of the end of the coding region (GenBank Accession Number Y08736).

A

```

nonameric
instability          Poly A          instability
                                Poly A
5' AAAGTGTTTTATATACGGTACTTATTTAATATCCCTTTTAAATTAGAAATTAAAACAGTTAATTTAATTAAAGATAGGGTTTTTTTCA
                                     (SEQ. ID NO:7)

```

B

```

instability          nonameric
                    instability
5' TTTTAAATTTAATATTTGTTATCATTTATTTATTGGTGCTACTGTTTATCCGTAATAATT (SEQ. ID NO:8)

```

f) Poly A Sites

The instability sequences appear to be closely associated with the poly A sequences, of which four have been identified in VEGF. These are positioned at 388, 1250, 1268 and 1891 bases downstream of the end of the VEGF coding region (GenBank Accession Number Y08736). The most commonly used poly A site has been defined as the site furthest from the end of the coding region which results in a 3.7kb mRNA product (Levy et al, 1995). Targeting of oligonucleotides spanning and adjacent to these potential poly A sites, with particular emphasis on the site at 1891, might influence the stability of the mRNA products. The sequences surrounding these four sites are shown below:

388 Position:

```

instability          Poly A          instability
5' ATACATTTATATATATATATATTATATATATATAAAATAATATCTCTATTTTATATATATATAAAATATATA (SEQ. ID NO:9)

```

1250 and 1268 Positions:

nonameric
instability

Poly A

instability
Poly A

5' AAAGTGTTTTATATACGGTACTTATTTAATATCCCTTTTAAATTAGAAATTAAACAGTTAATTTAATTAAAGATAGGGTTTTTTTCA
(SEQ. ID NO:10)

1891 Position:

Poly A instability

5' TCTTAAAAAAAAAAGCATTTTGTATTAAAGAATTAAATTCTGATCTCAAAGCTCCTCTT (SEQ. ID NO:11)

Oligonucleotides are designed in an anti-sense format that will bind directly with the mRNA and potentially alter the secondary structure or prevent binding of trans acting factors.

It will be appreciated that the present invention is particularly useful in the study, treatment or prevention of age-related macular degeneration, by virtue of the successful adenoviral gene transfer to the RPE. Without wishing to be bound by any proposed mechanism for the observed advantages, the higher degree of gene expression in the HRPE7 cells, compared with the F2000.]